

## **REMARKS**

Claims 3, 13, 21-44, 46, 54-55, 58-60 and 241-247 were pending before entry of the present amendment. Claims 248-250 have been newly added. Support for newly added claims 248-250 can be found in the specification as originally filed at ¶ 22, ¶¶ 240-241, ¶¶ 246-250 and in claims 4-12, respectively. No new matter has been introduced. Claims 3, 13, 21-44, 46, 55, 58-60 and 241-250 will be pending upon entry of the present amendment.

### **THE REJECTION FOR NONSTATUTORY OBVIOUSNESS-TYPE DOUBLE PATENTING SHOULD BE HELD IN ABEYANCE**

As this double patenting rejection is a provisional rejection, Applicants will not address this rejection at this time and request that the double patenting rejection be held in abeyance.

### **THE REJECTION UNDER 35 U.S.C. § 102(b) SHOULD BE WITHDRAWN**

Claims 46, 54, 55 and 241-247 were rejected under 35 U.S.C. § 102(a) as being allegedly anticipated by Lu *et al.* (Journal of Virology, 2002, 76(6):2871-2880; "Lu"). Lu, however, is not available as prior art against the claimed invention. Lu describes Applicants' own work (M.P.E.P. 715.01(d)) as it was co-authored by Hong Jin, Bin Lu, and Xing Cheng, three of the four currently listed inventors. To support this contention, Applicants provide a Declaration Under 37 C.F.R. § 1.132 ("Declaration") as evidence that Lu is the publication of the inventors which occurred less than one year prior to the effective filing date of the present application. *In re Katz*, 687 F.2d 450, 215 U.S.P.Q. 14 (C.C.P.A. 1982). The Declaration is executed by the actual inventors. Applicants submit herewith a Request to Correct Inventorship under 37 C.F.R. § 1.48(a) to include the name of Robert Brazas as inventor of the claims in instant application and to remove the names of Xing Cheng and Helen Zhou who were erroneously listed as co-inventors.

Since Lu is not available as prior art for any purpose under 35 U.S.C. § 102, Applicants request that Lu be withdrawn as prior art against the present application.

## **THE REJECTION UNDER 35 U.S.C. § 102(b) SHOULD BE WITHDRAWN**

Claims 46, 54, 55, 241, 242 and 247 were rejected under 35 U.S.C. § 102(b) as being allegedly anticipated by Marriott *et al.* (Journal of Virology, 1999, 73(6):5162-5165; "Marriott"). Applicants disagree as set forth in detail below.

### **THE LEGAL STANDARD**

Anticipation requires that the same invention, including each element and limitation of the claims, was known or used by others before it was invented by the patentee. *Hoover Group, Inc. v. Custom Metalcraft, Inc.*, 66 F. 3d 299, 302 (Fed. Cir. 1995). An anticipating reference must describe and enable the claimed invention, including all the claim limitations, with sufficient clarity and detail to establish that the subject matter already existed in the prior art and that its existence was recognized by persons of ordinary skill in the field of the invention. *In re Spada*, 911 F.2d 705 (Fed. Cir. 1990); *Crown Operations International, Ltd. v. Solutia Inc.*, 289 F.3d 1367, 1375 (Fed. Cir. 2002). Thus, a single prior art reference must teach all the claim limitations.

### **MARRIOTT DOES NOT TEACH ALL THE CLAIM LIMITATIONS**

The presently pending claims are directed to nucleic acids encoding the genome of a recombinant replicating respiratory syncytial viruses having an attenuated phenotype and in which the phosphoprotein comprises at least one mutated amino acid residue.

Marriott describes experiments using an earlier identified allele of the P gene. This allele is encoded by the genome of the previously discovered mutant RSV *tsN19* (Caravokyri et al., J. Gen. Virol. 1992, 73:865-873; Caravokyri et al., Virus Genes 1992, 6:53-62). To identify the underlying mutation in *tsN19*, total RNA was purified from cells infected with either wild-type or mutant virus and used as a template for reverse transcription using P gene-specific primers (Caravokyri et al., Virus Genes 1992, 6:53-62). Only the P gene mRNA was reverse-transcribed, amplified, and then sequenced. A mutation resulting in a glycine-to-serine substitution at position 172 ("G172S") was identified.

Marriott tested the effect of the G172S substitution in the P protein in the so-called plasmid-driven minigenome RNA transcription-replication assay ("minigenome assay"). In Marriott's minigenome assay, the four proteins of the RSV replication complex, the N, P, L, and M2 proteins, were co-expressed in a host cell. Once expressed, the RSV replication

complex transcribed RNA from a construct that included two reporter genes, chloramphenicol acetyltransferase (CAT) and luciferase (LUC), flanked by untranscribed regions of the RSV genome (“pCAT-LUC”). The activity of the RSV replication complex was then monitored using the activity of CAT and LUC as read-out. Using this assay system, Marriott found that the G172S substitution in the P protein results in thermosensitivity of the replication complex.

Marriott teaches four separate plasmids that contain the open reading frames (“ORFs”) of the RSV P protein, N protein, L protein, and M2 protein, respectively. Thus, Marriott does not disclose a nucleic acid encoding a viral genome, let alone an attenuated replicating virus. In contrast, the present claims are directed to an isolated nucleic acid encoding a replicating, attenuated RSV.

In summary, Marriott does not disclose a nucleic acid encoding the genome of a replicating, attenuated virus with a mutation in the P-gene. Thus, Marriott fails to teach all the limitations of the rejected claim. Applicants therefore request that the rejection of claims 46, 54, 55, 241, 242 and 247 under 35 U.S.C. § 102(b) should be withdrawn.

#### **THE REJECTION UNDER 35 U.S.C. § 103(a) SHOULD BE WITHDRAWN**

Claims 243-246 were rejected under 35 U.S.C. § 103(a) as being obvious over Marriott et al. (Journal of Virology, 1999, “Marriott”) in view of Khattar et al. (Journal of General Virology, 2001, “Khattar”) and Girault (Encyclopedia of Molecular Biology, 1999, “Girault”). Applicants disagree as set forth in detail below.

#### **THE LEGAL STANDARD**

To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the prior art references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Third, the prior art reference (or references when combined) must teach or suggest all the claim limitations. (M.P.E.P. 2143).

MARRIOTT, KHATTAR, AND GIRAULT DO NOT TEACH ALL THE  
ELEMENTS OF THE CLAIMED INVENTION

Both Marriott and Khattar employ minigenome systems to study different aspects of the RSV P protein. Neither Marriott or Khattar teaches a nucleic acid encoding a replicating, attenuated virus. Marriott describes separate plasmids containing the ORFs of P protein, N protein, L protein and M2 protein, respectively, but fails to describe a nucleic acid encoding a replicating RSV. Similarly, Khattar fails to teach a nucleic acid that encodes a replicating virus. Girault teaches the importance of protein phosphorylation in biological systems and fails to mention RSV. Therefore, neither Marriott, Khattar nor Girault, alone or in combination, teach all the elements of the claimed invention.

Thus, none of the prior art references alone or in combination teaches or suggests all the claim limitations of claims 243-246. Based on the arguments set forth above, Applicants request that the rejection of claims 243-246 under 35 U.S.C. § 103(a) should be withdrawn.

**CONCLUSION**

Applicants request that the present submission be entered into the file of the present application. Please contact the undersigned representative for Applicants with any questions regarding the above.

Date: February 4, 2008

Respectfully submitted,

by: *Jacqueline Bena*  
Reg No. 43,492

Laura A. Coruzzi 30,742  
Laura A. Coruzzi (Reg. No.)

**JONES DAY**  
222 East 41st Street  
New York, New York 10017  
(212) 326-3939

## Assignment of mutant *ts*N19 (complementation group E) of respiratory syncytial virus to the P protein gene

Calliope Caravokyri, Allan J. Zajac and Craig R. Pringle\*

Biological Sciences Department, University of Warwick, Coventry CV4 7AL, U.K.

The mutation responsible for the temperature-sensitive (*ts*) phenotype of mutant *ts*N19 (complementation group E) of respiratory syncytial virus has been located to the P protein gene. Viral protein synthesis was completely restricted at 39 °C, and the *ts*N19 P protein did not react with an anti-P monoclonal antibody (MAb) (3-5) at 33 °C. Reversion of temperature sensitivity restored reactivity with MAb 3-5. Nucleotide sequence determination and *in vitro* expression of

cDNA clones of P mRNA derived from wild-type, *ts*N19 and non-*ts* revertant-infected cells, revealed that temperature sensitivity and loss of reactivity with MAb 3-5 were consequences of a Gly → Ser amino acid change at position 172. A low *M<sub>r</sub>* polypeptide, which represented the C-terminal 93 amino acids of the P protein, was produced by internal initiation in the P open reading frame during *in vitro* translation, and a similar product was detected transiently *in vivo*.

### Introduction

The genome of respiratory syncytial (RS) virus comprises 10 genes ordered in a linear sequence from a 3'-terminal promoter with a single 68 nucleotide overlap of the end of the penultimate 22K protein gene and the start of the 5'-proximal L protein gene (Huang & Wertz, 1982; Collins & Wertz, 1983; Dickens *et al.*, 1984; Collins *et al.*, 1986, 1987). Eight complementation groups of temperature-sensitive (*ts*) mutants have been described which are presumed to correspond to eight of these 10 transcriptional units (Wright *et al.*, 1973; Gimenez & Pringle, 1978; Pringle *et al.*, 1981). Assignment of these complementation groups will assist analysis of gene function. Previously we have presented evidence for assignment of complementation groups B and D to the attachment (G) and matrix (M) protein genes respectively (Caravokyri & Pringle, 1991). Here we describe evidence for assignment of a third complementation group.

### Methods

**Viruses and cells.** The A2 wild-type strain (subgroup A) was obtained originally from Dr R. M. Chanock. The origin of the RSN-2 wild-type strain (subgroup B) and mutant *ts*N19 has been described previously (Faulkner *et al.*, 1976). The mutant, wild-type and non-*ts* revertant viruses were propagated in BS-C-1 cells maintained in the Glasgow modification of Eagle's medium supplemented with 2% foetal calf serum and antibiotics. Infectivity assays were carried out in CO<sub>2</sub> incubators. Radiolabelling experiments were carried out in small flasks totally immersed in a precision water bath. The permissive temperature was 33 °C and the restrictive temperature 39 °C. Non-*ts* revertants

were isolated by propagation of virus from the few plaques which appeared on *ts*N19-inoculated plates after incubation for 5 to 7 days at 39 °C.

**Monoclonal (MAb) and polyclonal antibodies.** The polyclonal bovine anti-RS virus serum was provided by Dr Geraldine Taylor (Institute of Animal Health, Compton, U.K.) and had been prepared by immunization of a gnotobiotic calf. The anti-P protein MABs 8268, 9178 and 9516, and the anti-N MABs N2 and N7 were obtained from Dr C. Örvell (Karolinska Institute, Stockholm, Sweden), and the anti-P MABs 3-5 and 4-14 from Dr Beatriz Gimenez (University of Aberdeen, U.K.).

**Radiolabelling, immunoprecipitation and SDS-PAGE.** Standard methods were employed (Caravokyri & Pringle, 1991).

**cDNA cloning and nucleotide sequencing.** cDNA clones of P mRNA were produced by reverse transcription followed by polymerase chain reaction (PCR) amplification and were sequenced by the dideoxynucleotide chain termination method (Caravokyri & Pringle, 1991).

**In vitro protein synthesis.** Total cytoplasmic RNA from infected or uninfected cells (up to 10 µg) or uncapped RNA transcripts produced *in vitro* (150 to 300 ng) were translated in a nuclease-treated rabbit reticulocyte lysate (NEN) as previously described (Caravokyri & Pringle, 1991, 1992).

### Results

#### *Viral protein synthesis*

Previously, cells infected by mutant *ts*N19 (RSN-2 strain) were found to be negative for both intracellular and surface immunofluorescent staining (Pringle *et al.*, 1981), suggesting that the *ts*N19 defect was exhibited

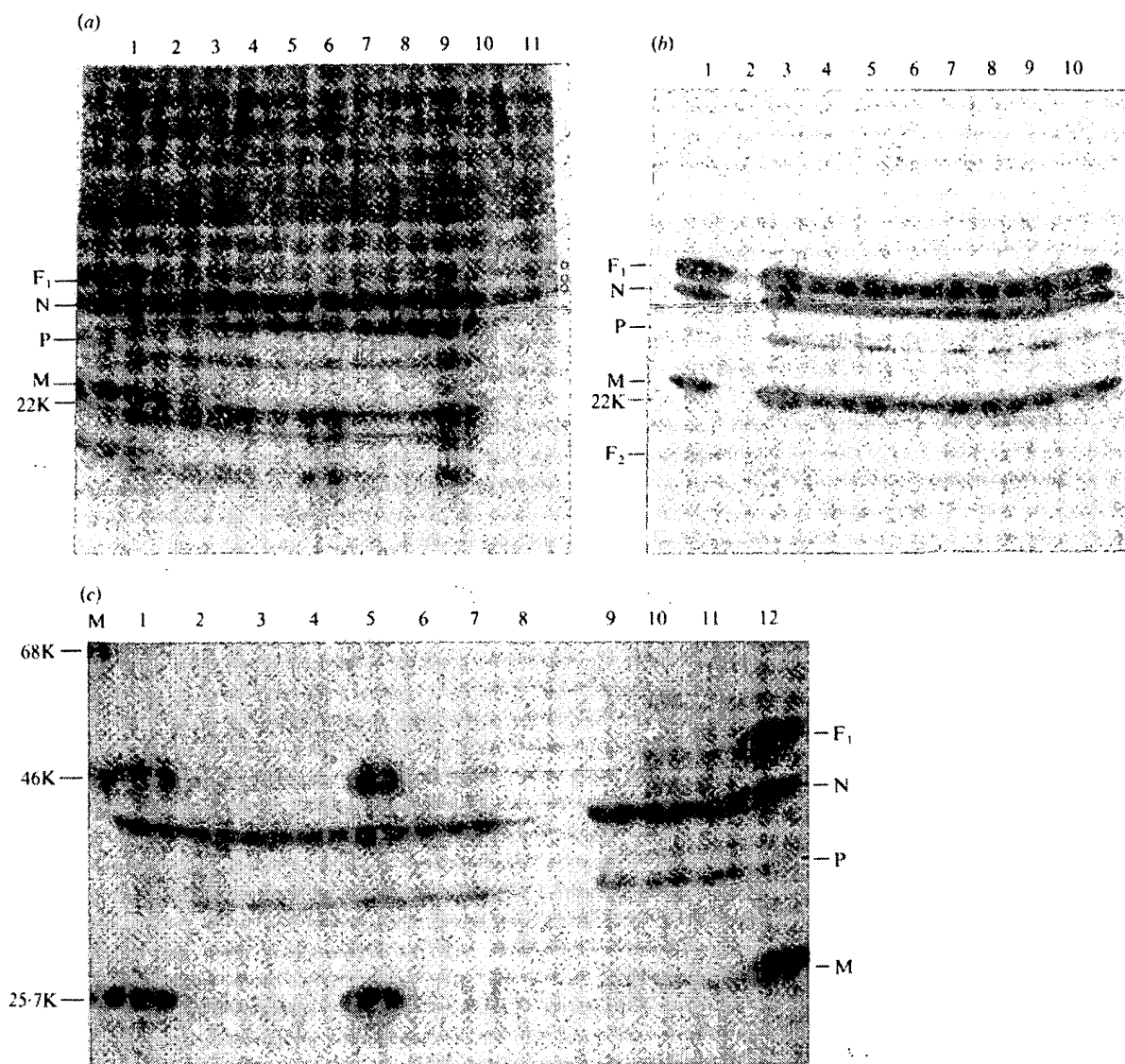


Fig. 1. Viral polypeptide synthesis by mutant *ts*N19 and three non-*ts* revertants. (a) BS-C-1 cells were infected with wild-type RSN-2 (lanes 1 and 2), mutant *ts*N19 (lanes 9 and 10) or three different non-*ts* revertant clones [R3/1 (lanes 3 and 4), R3/4 (lanes 5 and 6) and R3/6 (lanes 7 and 8)]. Cells were incubated at 33 °C (lanes 1, 3, 5, 7 and 9) and 39 °C (lanes 2, 4, 6, 8, 10 and 11). Infected and mock-infected (lane 11) cell monolayers were radiolabelled with [<sup>35</sup>S]methionine, lysed and immunoprecipitated with polyclonal antiserum. (b) Virus-specific polypeptides were resolved in 6 to 15% gradient gels. BS-C-1 cells were infected with mutant *ts*N19 (lanes 1 and 2), non-*ts* revertant clones [as for (a) lanes 3 to 8] and wild-type RSN-2 (lanes 9 and 10). Released virus was recovered by polyethylene glycol precipitation and analysed similarly. (c) Released virus [wild-type RSN-2 (lanes 1 to 4), *ts*N19 (lanes 5 to 8) and R3/6 (lanes 9 to 12)] in the supernatants of cells infected and labelled at 33 °C was immunoprecipitated with either polyclonal serum (lanes 1), or the anti-P MABs 8268 (lanes 2), 9178 (lanes 3) or 3-5 (lanes 4). Protein M, markers are shown in lane M; F<sub>1</sub>, N, P and M proteins are indicated.

early in infection. This result is confirmed by the experiment illustrated in Fig. 1. BS-C-1 cell cultures were infected with mutant *ts*N19, a non-*ts* revertant or wild-type virus, and incubated at permissive (33 °C) or restrictive (39 °C) temperatures and radiolabelled with [<sup>35</sup>S]methionine as described previously (Caravokyri & Pringle, 1991). No intracellular or virion-associated polypeptides could be detected in *ts*N19-infected cul-

tures incubated at 39 °C (Fig. 1 *a* and *b*, respectively). In contrast, non-*ts* revertants isolated from *ts*N19 stocks exhibited efficient viral protein synthesis at 39 °C and their profiles were indistinguishable from that of the RSN-2 wild-type virus.

The P protein of mutant *ts*N19 did not react with anti-P MAB 3-5 after infection and radiolabelling at the permissive temperature of 33 °C (Fig. 1 *c*). This negative

reaction has also been observed by Western blotting and by immunofluorescence (B. Gimenez, personal communication). The *tsN19* P protein was found to react with all other anti-P MAbs tested (two of which are shown in Fig. 1c), including MAbs representing the three B subgroup epitopes defined by Örvell *et al.* (1987). The P protein of revertant clone R3/6 (Fig. 1c) and of another five independently isolated revertants (not shown) was immunoprecipitated by anti-P MAb 3-5. The simultaneous reversion of the *ts* phenotype and restoration of reactivity of the P protein with MAb 3-5 in the non-*ts* revertants indicated that the P protein of mutant *tsN19* was the site of its *ts* lesion.

The intracellular stability at 39 °C of the P protein of *tsN19* presynthesized at 33 °C was examined by pulse-radiolabelling at 33 °C followed by a chase period at 39 °C. Fig. 2 shows the negative profile of *tsN19*-infected cultures incubated at 39 °C immediately post-infection (lane 1). However, when *tsN19*-infected cells that had been maintained and labelled at 33 °C were chased at 39 °C for up to 2 h, no decrease of the P protein band was observed. In another experiment BS-C-1 cells were infected with wild-type virus, mutant *tsN19* or mutant *tsN1* which expresses a thermolabile M protein (Caravokyri & Pringle, 1991), and shifted up to 39 °C after 24 h incubation at 33 °C. The yields of infectious virus from the *tsN19*-infected cultures after 24 h at 39 °C were similar to those from wild-type virus-infected cultures, whereas the yields from *tsN1*-infected cultures showed no enhancement over the yields obtained from replicate cultures maintained at 33 °C throughout (data not shown). These results established that the *tsN19* protein presynthesized at 33 °C was not thermolabile and that it retained its functional activity at 39 °C.

#### Production and sequencing of P cDNA clones

In order to identify the site of the *tsN19* lesion at the nucleotide level, cDNA clones of the P mRNA were produced as described elsewhere (Caravokyri & Pringle, 1992). Two different clones from each type of viral mRNA (i.e. wild-type, non-*ts* revertant or *tsN19*), representing the two opposite orientations of insertion into the plasmid multiple cloning site, were selected for sequence determination. The nucleotide differences found in each clone relative to the consensus RSN-2 sequence (GenBank accession number M67450; Caravokyri & Pringle, 1992) are listed in Table 1. These differences could be artefacts of the *in vitro* synthetic process and/or due to variation in the original mRNA population. Indeed, the greater nucleotide variability seen in the two *tsN19*-derived clones, compared with the revertant clones which were derived from more recently plaque-purified virus, may indicate greater mRNA

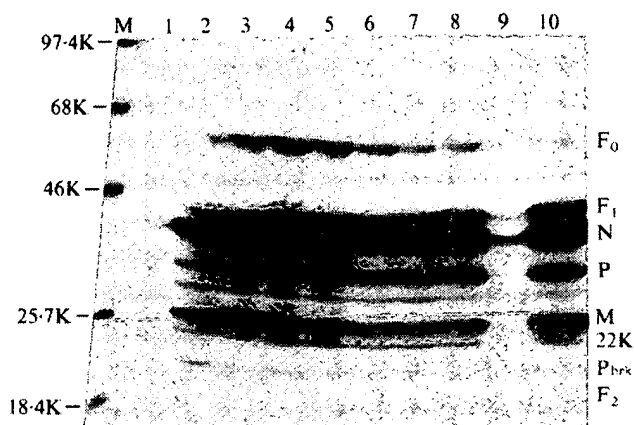


Fig. 2. Intracellular stability of the *tsN19* P protein. Replicate BS-C-1 cell monolayers were infected with *tsN19* and incubated at 33 °C or 39 °C for 48 h, then pulse-labelled with [<sup>35</sup>S]methionine for 10 min. One culture from each temperature was lysed and viral proteins were immunoprecipitated with polyclonal antiserum immediately after removal of the radiolabel (lanes 1, 39 °C and lane 2, 33 °C). The remaining cultures were similarly treated after further incubation in medium containing non-radioactive methionine for periods up to 120 min (10, 20, 30, 45, 60 and 120 min; lanes 3 to 8, respectively). One set of cultures was shifted from 33 °C to 39 °C after pulse labelling (lanes 3 to 8). Control cultures were maintained throughout at 33 °C (lane 10) or 39 °C (lane 9). *M<sub>r</sub>* markers are shown in lane M. Pbrk is a breakdown product of the P protein.

heterogeneity of the mutant stock. However, of the three or four substitutions identified in the two clones from mutant *tsN19*, only the G → A substitution at position 531 was common to both clones. Furthermore this substitution caused the only predicted amino acid change in one of the clones (N23), indicating that this is the site of the *tsN19* mutation.

#### In vitro synthesis of the P protein

To confirm that the Gly → Ser change (predicted from the sequence of the two mutant clones) correlated with the loss of the epitope defined by MAb 3-5, it was necessary to use this antibody for immunoprecipitation of P proteins expressed from the PCR-derived clones. Therefore the P cDNA clones were transcribed *in vitro* and the P mRNA-sense transcripts were used to programme protein synthesis in the rabbit reticulocyte lysate system. Both the *tsN19*-derived cDNA clones produced a P-specific protein which comigrated with the *in vitro* translated *tsN19* P protein (Fig. 3). Also both the P proteins from revertant clone R34 (Ile → Val) and the mutant clone N23 (Gly → Ser) were immunoprecipitated with anti-P MAb 8268. However, in contrast to the R34 P protein, the N23 P protein which contained the single

Table 1. Nucleotide differences of individual P cDNA clones and predicted changes in their respective proteins

cDNA clone	Virus origin	Nucleotide		Amino acid	
		Position	Substitution	Position	Substitution
N19	tsN19	297	A→G	94	Ser→Gly
		318	T→C	101	None
		531	G→A	172	Gly→Ser
		592	T→C	192	Leu→Ser
N23	tsN19	473	A→G	152	None
		531	G→A	172	Gly→Ser
		647	C→G	270	None
R29	ts <sup>+</sup> R3/6 (non-ts revertant of tsN19)	224	T→C	69	None
R34	ts <sup>+</sup> R3/6 (non-ts revertant of tsN19)	513	A→G	166	Ile→Val

Gly → Ser change did not react with MAb 3-5 (data not shown). Therefore, the Gly → Ser change at position 172 in the P protein of clone N23 seems to be solely responsible for the negative reaction to anti-P MAb 3-5. Since the same phenotype was observed *in vivo*, and recovery of the 3-5 epitope was coordinate with restoration of the non-ts phenotype in the revertants, the Gly → Ser change at amino acid position 172 of the P protein is the likely site of the ts lesion in mutant tsN19.

#### Internal initiation of translation in the P open reading frame (ORF)

Among the *in vitro* translation products of RNA transcripts produced from the P cDNA clones, a low  $M_r$  band of approximately 15K to 16K was invariably detected. Although additional (fainter) bands in the profiles could be correlated with similar bands present in *in vitro* translations of P mRNA from infected cells (probably reflecting some proteolytic degradation), only trace amounts of this low  $M_r$  band could be detected by *in vitro* translation of P mRNA synthesized *in vivo* (Fig. 3). This small polypeptide designated Pint was also translated from RNA produced by T7 *in vitro* transcription of a truncated A2 strain P cDNA insert in a different vector (pTZ19; Pharmacia). This cDNA clone (obtained from Dr G. Wertz) contained the full 3' non-coding P mRNA region, but was lacking the first 231 nucleotides of the P mRNA, including the coding sequence for the N-terminal 72 amino acids. The pTZ19/A2-P clone which produced P mRNA-sense transcripts from the T7 promoter could also be translated *in vitro* into the

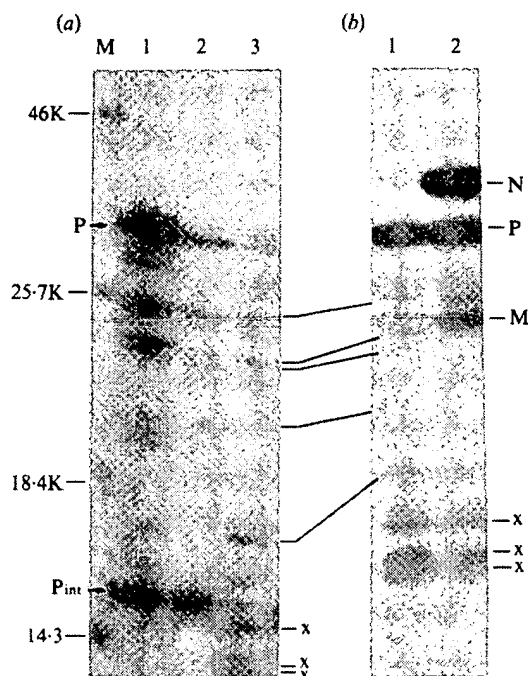


Fig. 3. *In vitro* translation of RNA from virus-infected cells and of transcripts from P cDNA clones. Translation products were immunoprecipitated with anti-P MAb 8628 (a and b, lane 1) or polyclonal anti-RS virus serum (b, lane 2) and analysed by SDS-PAGE. In (a), the profiles are from *in vitro* translated P transcripts of the two tsN19-derived cDNA clones N23 (lane 1) and N19 (lane 2), and from RNA isolated from tsN19-infected cells (lane 3); lane M contained  $M_r$  protein markers. Pint is the internally initiated product from the P ORF. In (b) both lanes contain *in vitro* translated RS virus proteins from tsN19-infected cell RNA. The bars between panels indicate corresponding products possibly originating by proteolytic breakdown, and the bands marked X are host cell-derived.

products shown in Fig. 4a, despite the absence of the authentic P start codon. As shown in Fig. 4(b) the initiator AUG is provided by the *SphI* site upstream from the *PstI* site of insertion. The resulting polypeptide is 171 amino acids in length (predicted  $M_r$  of 19.3K) and contains two amino acid differences from the authentic P protein. Two major P-specific bands (recognized by polyclonal serum and anti-P MAb 3-5; Fig. 4a), with apparent mobilities of 21K and 23K, were observed. These probably represent the N-terminally truncated forms of the P protein of the A2 wild-type, which migrates as a doublet band (Fig. 5a and c). Two faster migrating, fainter bands (possibly proteolytic fragments) are also present in both the polyclonal and anti-P MAb profiles (indicated by arrows); however, the abundant low  $M_r$  polypeptide was not immunoprecipitated by anti-P MAb 3-5. A fainter band of similar mobility to the low  $M_r$  polypeptide can be observed also in the A2 strain profile, suggesting that this product is not an artefact of the *in vitro* expression of the P protein from cloned



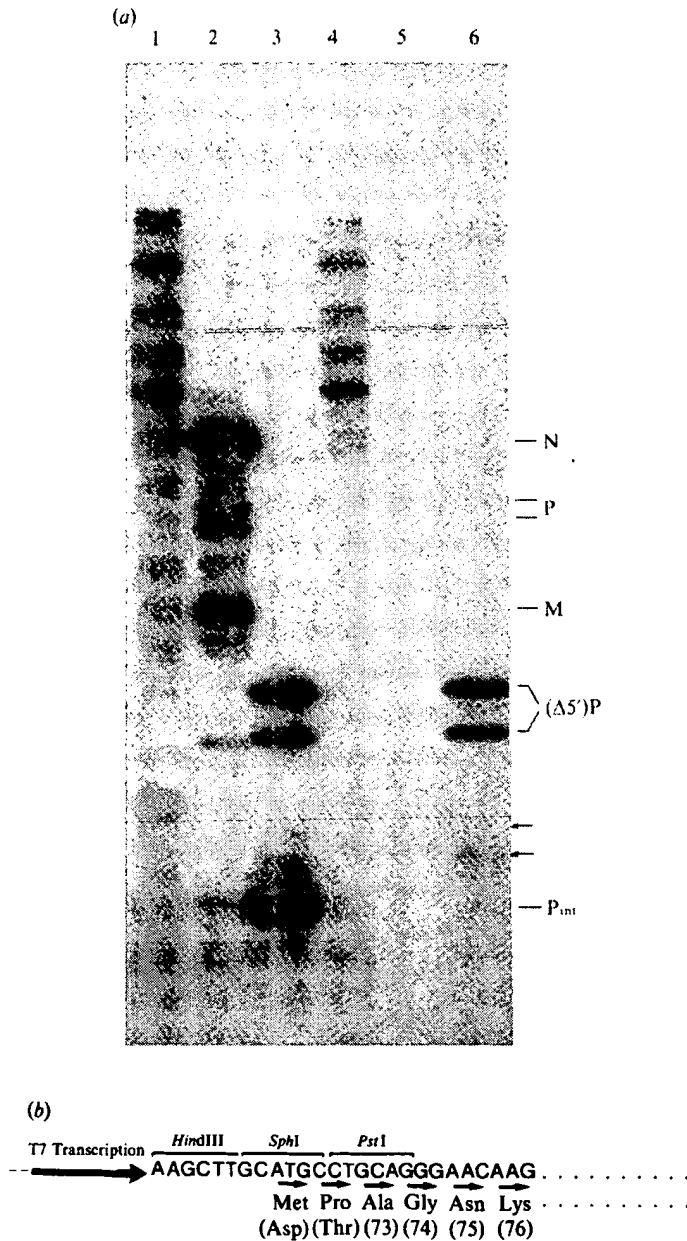


Fig. 4. *In vitro* synthesis of an N-terminally truncated form of the P protein. (a) The *in vitro* translation products of cytoplasmic RNA synthesized in mock-infected cells (lane 1), A2 wild-type-infected cells (lane 2), and of the transcripts synthesized *in vitro* from recombinant vectors pTZ18/P (– sense; lanes 4 and 5) and pTZ19/P (+ sense; lanes 3 and 6). Recombinant plasmid pTZ18/P produces T7 transcripts of the truncated P cDNA in the antisense orientation. The translation products were immunoprecipitated with polyclonal antiserum (lanes 1 to 4) or MAb 3-5 (lanes 5 and 6). The N-terminally truncated form of the A2 strain doublet is indicated [(Δ5')P]. Pint is the internally initiated product from the P ORF. (b) The sequence at the site of ligation of the 5'-end of the truncated P cDNA clone with the *Pst*I site of plasmid pTZ19. (The truncated clone contains two terminal *Pst*I sites; the 5' site is internal in the P cDNA sequence at residue 232.) *In vitro* transcription of the recombinant plasmid pTZ19/P from the adjacent T7 promoter produced P mRNA sense transcripts (having the same sequence shown here as DNA). The in-frame start codon is

cDNA. The P specificity of this product was also confirmed by its immunoprecipitation by anti-P MAb 8268 (Fig. 5a). Venkatesan *et al.* (1984) also observed a similar (about 16K) band by *in vitro* translation of P cDNA hybrid-selected mRNA from infected cells that also translated the P protein.

The size and relative abundance of this product suggested either that significant proteolytic cleavage occurred at a specific site of the P protein in the absence of other viral proteins, or that translation from an internal start codon occurred in the same ORF as that of the P protein, since this product reacted with anti-P MAb 8268 (Fig. 5a).

The first in-frame AUG codon in the A2 strain P protein sequence (downstream of the initiating methionine in both the A2 and RSN-2 P proteins) is located at position 148 and surrounded by the sequence GGAAUGC, which contains a purine (G) at the –3 position. Presence of a purine in this position is considered to provide a favourable context for translational initiation (Kozak, 1986b, 1989a). To test the possibility of internal initiation at this site, a 5' *Bcl*I–*Pst*I 3' fragment of the A2 P clone (which contained only the AUG 148 as the first start codon approximately 80 bp from the 5' end) was subcloned into the transcription vector pGEM1 (Promega; Fig. 5b). Fig. 5 (c) shows that the translation product of T7 RNA transcripts from the subcloned P fragment comigrated with the abundant low  $M_r$  protein product of RNAs from the larger A2 P clone, suggesting that this polypeptide was produced by initiation of translation at AUG 148 rather than by proteolytic breakdown.

Since a number of experimental conditions (e.g. absence of a 5' cap, RNA degradation) can contribute to *in vitro* internal initiation of translation, synthesis of the Pint product was also investigated *in vivo*, by pulse-chase labelling of wild-type A2-infected cells and immunoprecipitation with anti-P MAb 8268. As shown in Fig. 6(b), a 16K band was detected during the 5 min pulse which disappeared during the 20 min chase period. In a similar experiment with strain RSN-2 (not shown), this low  $M_r$  band had the characteristically slower electrophoretic mobility noticed previously for the Pint product of this strain (Caravokyri & Pringle, 1992). These data suggest that as in some other negative-strand RNA viruses internal initiation of translation also occurs in the P mRNA of RS virus.

provided by the sequence of the immediately upstream *Sph*I restriction site. The numbers in parentheses denote amino acid positions in the intact A2 strain P protein. Residues 71 and 72 are replaced by Met and Pro in the truncated protein.

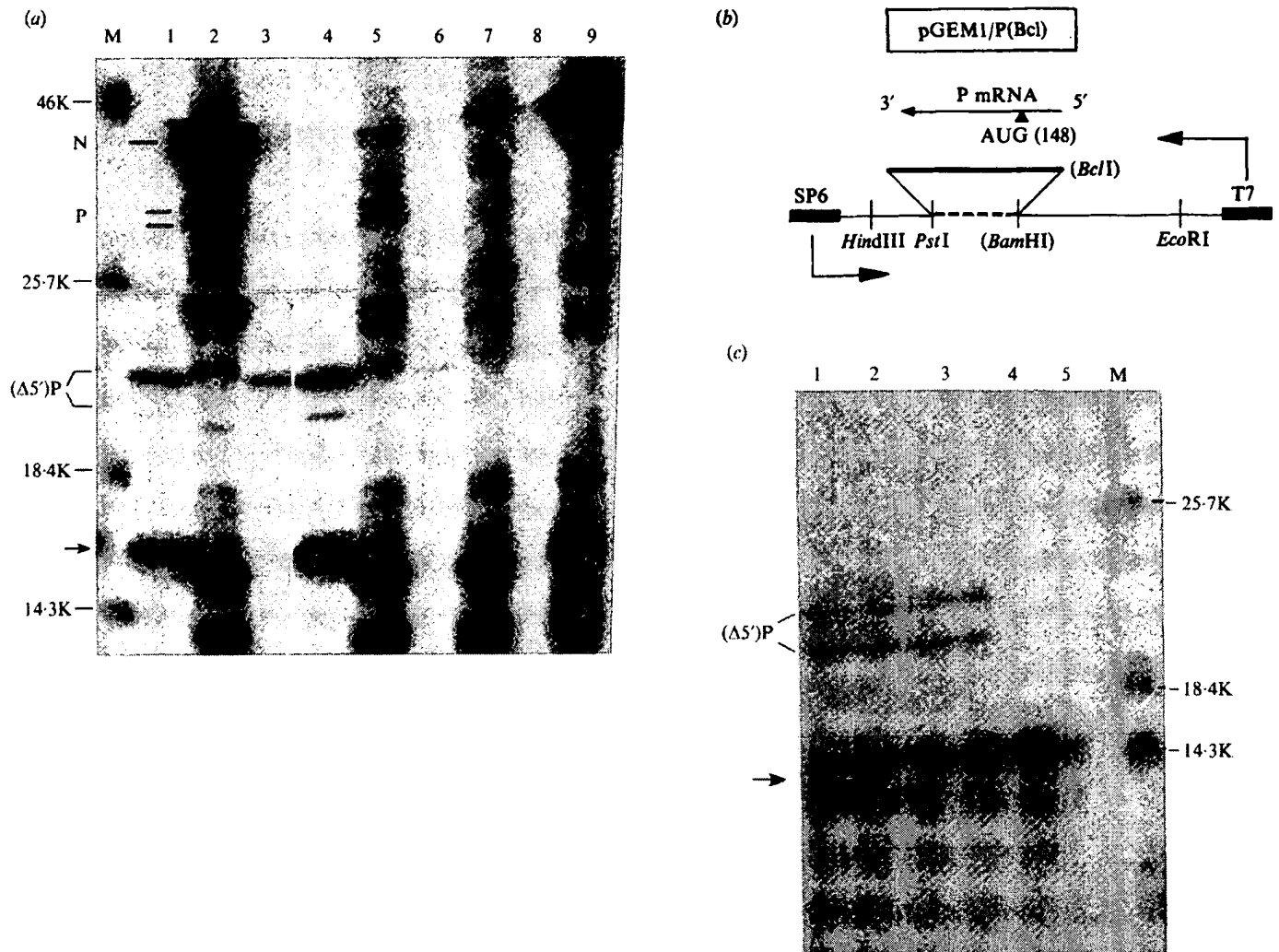


Fig. 5. Internal initiation in the A2 strain P ORF. (a) *In vitro* translation products of cytoplasmic RNA extracted from A2-infected cells (lanes 2, 5, 7 and 9), or *in vitro* synthesized RNA transcripts of the 5' end truncated p cDNA clone (lanes 1, 3, 4, 6 and 8) were immunoprecipitated with polyclonal anti-RS virus serum (lanes 1 and 2) or anti-P MAb 3-5 (lane 3), 8268 (lanes 4 and 5) and 9516 (lanes 6 and 7), or the anti-N MAb N2 (lanes 8 and 9), and analysed by 12.5% SDS-PAGE. The arrow indicates the position of the low  $M_r$  polypeptide which reacts with MAb 8268.  $M_r$  markers are shown (lane M). (b) A fragment of the P cDNA clone, containing an AUG codon approximately 80 bp from its 5' end (residue 148 of the complete P protein), was subcloned into the multiple cloning site (MCS) of vector pGEM1 which is flanked by the SP6 and T7 promoters (the arrows indicate the direction of transcription). The MCS region between the *Pst*I and *Bam*HI sites replaced by the cDNA insert in the recombinant plasmid is shown as a dashed line. The position of the internal AUG is marked by the arrowhead on the P mRNA sense T7 transcript. (c) *In vitro* translation products obtained from recombinant plasmid pTZ19/P (lanes 1 to 3), and from plasmid pGEM1/P(Bcl) by T7 transcription (lane 4) and by SP6 transcription (lane 5). The polypeptides were immunoprecipitated with anti-RS virus polyclonal serum and analysed by 15% SDS-PAGE. The arrow indicates the polypeptide translated from the internal AUG.  $M_r$  markers are shown (lane M).

## Discussion

Initially mutant *ts*N19 was associated with a P protein defect on the basis of coordinate reversion of the *ts* phenotype and restoration of a P protein epitope recognized by MAb 3-5. Nucleotide sequencing of the mutant and a non-*ts* revertant has shown that this phenotype is the result of a Gly → Ser replacement in the

predicted polypeptide sequence. Complementation group E, therefore, corresponds to the P gene of RS virus. Previously, complementation groups B and D have been assigned to the G and M genes respectively (Caravokyri & Pringle, 1991).

The paramyxovirus P and the rhabdovirus NS phosphoproteins are known to be essential components of the viral RNA polymerase complex (Emerson & Yu,

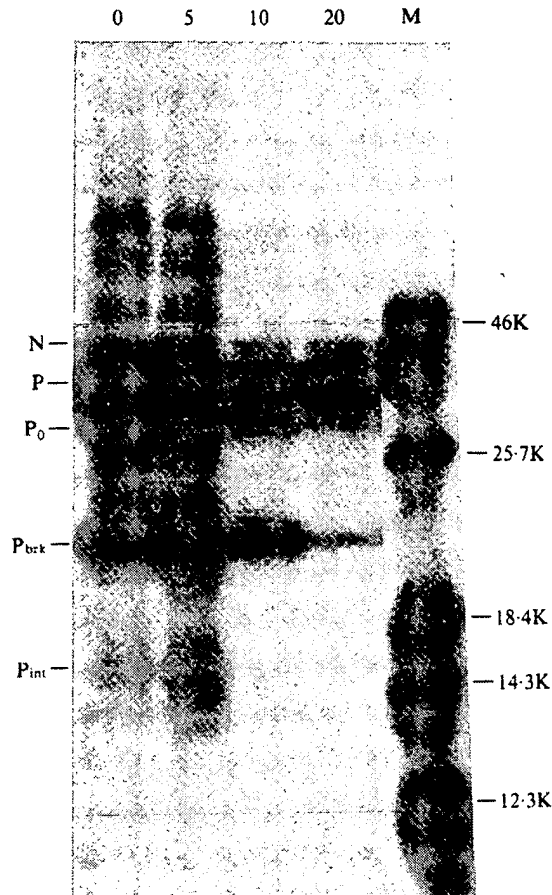


Fig. 6. Internal initiation of translation in the A2 P mRNA *in vivo*. Replicate monolayers of BS-C-1 cells, infected with RS virus strain A2 incubated at 33 °C for 48 h, then pulse-labelled with [ $^{35}$ S]methionine for 5 min. One culture was lysed and immunoprecipitated with anti-P MAb 8268 at the end of the pulse (lane 0). The remaining cultures were similarly treated after the chase periods indicated (min). P<sub>0</sub> is the unphosphorylated form of the P protein (Cash, 1978), Pbrk is a breakdown product of the P protein, and Pint is the internal initiation product. Lane M contains protein M<sub>r</sub> markers.

1975; Hamaguchi *et al.*, 1983; De & Banerjee, 1985; Deshpande & Portner, 1985). Consequently, ts lesions in these proteins have profound effects on viral mRNA synthesis at the restrictive temperature (Evans *et al.*, 1979; Peebles *et al.*, 1982). Early growth restriction of tsN19, as indicated by the absence of detectable protein synthesis at 39 °C, may reflect absence of synthesis of mRNA species.

Since the P protein of tsN19 presynthesized at 33 °C was stable at 39 °C, the negative phenotype at the restrictive temperature may be mediated by an effect on the functional configuration of the P protein. Direct reversion to the wild-type residue in revertant R3/6 suggests that the presence of glycine at position 172 may

be essential for the structural and/or functional integrity of the P protein.

The Gly → Ser change which abolishes reactivity with MAb 3-5 is located within the Pint region, but the Pint product was not immunoprecipitated by this MAb. In conjunction with the positive reaction of MAb 3-5 with the N-terminally truncated A2 P protein ( $\Delta 5'$ -A2) this observation indicates that residues and/or correct folding in both the middle and C-terminal thirds of the protein are required for MAb binding. Therefore the 3-5 epitope may be discontinuous. Although MAb 3-5 has the ability to react with the P protein on Western blots, a property often associated with recognition of denaturation-resistant (i.e. conformation-independent) antigenic determinants (reviewed by Lenstra *et al.*, 1990), the P protein of non-segmented negative-strand RNA viruses is thought to bind poorly to SDS due to electrostatic repulsion by its acidic and phosphate groups (Gallione *et al.*, 1981; Marnell & Summers, 1984). It is possible that binding of MAb 3-5 to P protein on Western blots is due to the inability of SDS to disrupt the conformation of the P protein effectively and/or the ability of partially denatured P protein to refold after boiling in SDS and transfer to a nitrocellulose membrane in the absence of detergent (Dunn, 1986).

Internal initiation of *in vitro* translation has been detected frequently in the rabbit reticulocyte lysate system, with frequencies depending on reaction conditions and RNA integrity (Gupta & Kingsbury, 1985; Dasso & Jackson, 1989; Kozak, 1989a, b). The internal initiation product (Pint) identified here was translated more efficiently in the absence of an upstream AUG or in the presence of a preceding AUG in an unfavourable context for initiation, and less efficiently in the presence of the authentic P mRNA start codon which lies in the optimal context for initiation according to the rules of the leaky scanning model (Kozak, 1986a, b).

As both the *in vitro* produced P transcripts and P mRNA molecules isolated from infected cells contain the same optimal P start codon, the observed difference in efficiency of internal initiation between the two kinds of transcripts (Fig. 3) could be a consequence of their different structures. Although reticulocyte lysate ribosomes exhibit relatively cap-independent binding to 5' RNA termini (Lodish & Rose, 1977; Kozak, 1989a, b), a property reflected here by the ability to translate the *in vitro* synthesized uncapped P RNA transcripts, the simultaneous presence of the 5' cap and 5' proximal AUG codons in mRNA molecules produced *in vivo* would probably have increased the efficiency of initiation at this first AUG triplet (Kozak, 1980; Dasso & Jackson, 1989). It is also possible that the absence of the 3' non-coding region from the *in vitro* transcripts affects secondary RNA structure, a factor contributing to the

selection of translational initiation sites (Shioda *et al.*, 1986; Kozak, 1986c, 1989a, c; Alkhatib *et al.*, 1988). Although any or all of the above factors may contribute to an enhanced frequency of *in vitro* internal initiation, the immunoprecipitation of the Pint polypeptide from infected cells indicates that internal initiation from the 3'-proximal AUG 148 does take place *in vivo* (with approximately the same efficiency as observed with *in vitro* translated viral mRNA; Fig. 4a, lane 2). In both Sendai virus and vesicular stomatitis virus internal initiation at a 3'-proximal AUG codon in the phosphoprotein mRNA leads to synthesis of the X and 7K proteins, respectively (Curran & Kolakofsky, 1987, 1988a, b; Vidal *et al.*, 1988; Herman, 1986, 1987). Both polypeptides also represent the C-terminal regions of the respective phosphoproteins and have been detected in small amounts *in vivo*, although their functional roles remain unknown. The RS virus Pint product, consisting mainly of the acidic domains of the P protein, may play a role in infection by interacting with the basic N and/or 22K proteins.

We are indebted to Dr Gail Wertz for provision of the truncated P cDNA clone, and to Dr Beatriz Gimenez for generous cooperation and provision of reagents. This work was supported by MRC Programme Grant no. PG8322715, the WHO Vaccine Development Programme, and by a scholarship to C.C. from the Greek 'A. S. Onassis' Public Benefit Foundation.

## References

- ALKHATIB, G., MASSIE, B. & BRIEDIS, D. J. (1988). Expression of bicistronic measles virus P/C mRNA by using hybrid adenoviruses: levels of C protein synthesized *in vivo* are unaffected by the presence or absence of the upstream P initiator codon. *Journal of Virology* **69**, 4059-4069.
- CASH, P. (1978). *Characterisation of pneumovirus polypeptides*. Ph.D thesis, University of Glasgow.
- CARAVOKYRI, C. & PRINGLE, C. R. (1991). Defective synthesis of envelope proteins by temperature-sensitive mutants representing complementation groups B and D of respiratory syncytial virus. *Journal of General Virology* **72**, 2501-2508.
- CARAVOKYRI, C. & PRINGLE, C. R. (1992). Effect of changes in the nucleotide sequence of the P gene of respiratory syncytial virus on the electrophoretic mobility of the P protein. *Virus Genes* **6**, 53-62.
- COLLINS, P. L. & WERTZ, G. W. (1983). cDNA cloning and transcriptional mapping of nine polyadenylated RNAs encoded by the genome of human respiratory syncytial virus. *Proceedings of the National Academy of Sciences, U.S.A.* **80**, 3209-3212.
- COLLINS, P. L., DICKENS, L. E., BUCKLER-WHITE, A., OLMSTED, R. A., SPRIGGS, M. K., CAMARGO, E. & COELINGH, K. V. W. (1986). Nucleotide sequences for the gene junctions of human respiratory syncytial virus reveal distinctive features of intergenic structure and gene order. *Proceedings of the National Academy of Sciences, U.S.A.* **83**, 4594-4598.
- COLLINS, P. L., OLMSTED, R. A., SPRIGGS, M. K., JOHNSON, P. R. & BUCKLER-WHITE, A. (1987). Gene overlap and site-specific attenuation of transcription of the viral polymerase L gene of human respiratory syncytial virus. *Proceedings of the National Academy of Sciences, U.S.A.* **84**, 5134-5138.
- CURRAN, J. A. & KOLAKOFSKY, D. (1987). Identification of an additional Sendai virus non-structural protein encoded by the P/C mRNA. *Journal of General Virology* **68**, 2515-2519.
- CURRAN, J. A. & KOLAKOFSKY, D. (1988a). Ribosomal initiation from an ACG codon in the Sendai virus P/C mRNA. *EMBO Journal* **7**, 245-251.
- CURRAN, J. A. & KOLAKOFSKY, D. (1988b). Scanning-independent ribosomal initiation of the Sendai virus X protein. *EMBO Journal* **7**, 2869-2874.
- DASSO, M. C. & JACKSON, R. J. (1989). On the fidelity of mRNA translation in the nuclease-treated rabbit reticulocyte lysate system. *Nucleic Acids Research* **17**, 3129-3144.
- DE, B. P. & BANERJEE, A. K. (1985). Requirements and functions of vesicular stomatitis virus L and NS proteins in the transcription process *in vitro*. *Biochemical and Biophysical Research Communications* **126**, 40-49.
- DEHPANDE, K. L. & PORTNER, A. (1985). Monoclonal antibodies to the P protein of Sendai virus define its structure and role in transcription. *Virology* **140**, 125-134.
- DICKENS, L. E., COLLINS, P. L. & WERTZ, G. W. (1984). Transcriptional mapping of human respiratory syncytial virus. *Journal of Virology* **52**, 364-369.
- DUNN, S. D. (1986). Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on Western blots by monoclonal antibodies. *Analytical Biochemistry* **157**, 144-153.
- EMERSON, S. U. & YU, Y.-H. (1975). Both NS and L proteins are required for *in vitro* RNA synthesis by vesicular stomatitis virus. *Journal of Virology* **15**, 1348-1356.
- EVANS, D., PRINGLE, C. R. & SZILAGYI, J. F. (1979). Temperature-sensitive mutants of complementation group E vesicular stomatitis virus New Jersey serotype possess altered NS polypeptides. *Journal of Virology* **31**, 325-333.
- FAULKNER, G. P., SHIRODARIA, P. V., FOLLET, E. A. C. & PRINGLE, C. R. (1976). Respiratory syncytial virus *ts* mutants and nuclear immunofluorescence. *Journal of Virology* **20**, 487-500.
- GALLIONE, C. J., GREENE, J. R., IVERSON, L. E. & ROSE, J. K. (1981). Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus N and NS proteins. *Journal of Virology* **39**, 529-535.
- GIMENEZ, H. B. & PRINGLE, C. R. (1978). Seven complementation groups of respiratory syncytial virus temperature-sensitive mutants. *Journal of Virology* **27**, 459-464.
- GUPTA, K. C. & KINGSBURY, D. W. (1985). Translational modulation *in vitro* of a eukaryotic viral mRNA encoding overlapping genes: ribosome scanning and potential roles of conformational changes in the P/C mRNA of Sendai virus. *Biochemical and Biophysical Research Communications* **131**, 91-97.
- HAMAGUCHI, T., NISHIKAWA, K., NARUSE, H. & NAGAI, Y. (1983). Transcriptional complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. *Virology* **128**, 105-117.
- HERMAN, R. C. (1986). Internal initiation of translation on the vesicular stomatitis virus phosphoprotein mRNA yields a second protein. *Journal of Virology* **58**, 797-804.
- HERMAN, R. C. (1987). Characterization of the internal initiation of translation on the vesicular stomatitis virus phosphoprotein mRNA. *Biochemistry* **26**, 8346-8350.
- HUANG, Y. T. & WERTZ, G. W. (1982). The genome of respiratory syncytial virus is a negative-stranded RNA that codes for at least seven mRNA species. *Journal of Virology* **43**, 150-157.
- KOZAK, M. (1980). Binding of wheat germ ribosomes to fragmented viral mRNA. *Journal of Virology* **35**, 748-756.
- KOZAK, M. (1986a). Bifunctional messenger RNAs in eukaryotes. *Cell* **47**, 481-483.
- KOZAK, M. (1986b). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**, 283-292.
- KOZAK, M. (1986c). Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. *Proceedings of the National Academy of Sciences, U.S.A.* **83**, 2850-2854.

- KOZAK, M. (1989a). Context effects and inefficient initiation at non-AUG codons in eucaryotic cell-free translation systems. *Molecular and Cellular Biology* **9**, 5073–5080.
- KOZAK, M. (1989b). The scanning model for translation: an update. *Journal of Cell Biology* **108**, 229–241.
- KOZAK, M. (1989c). Circumstances and mechanisms of inhibition of translation by secondary structure in eukaryotic mRNAs. *Molecular and Cellular Biology* **9**, 5134–5142.
- LENSTRA, J. A., KUSTERS, J. G. & VAN DER ZEUST, B. A. M. (1990). Mapping of viral epitopes with prokaryotic expression products. *Archives of Virology* **110**, 1–24.
- LODISH, H. F. & ROSE, J. K. (1977). Relative importance of 7-methylguanosine in ribosome binding and translation of VSV mRNA in wheat germ and reticulocyte cell-free systems. *Journal of Biological Chemistry* **252**, 1181–1188.
- MARNELL, L. L. & SUMMERS, D. F. (1984). Characterization of the phosphorylated small enzyme subunit, NS, of the vesicular stomatitis virus RNA polymerase. *Journal of Biological Chemistry* **259**, 13518–13524.
- ÖRVELL, C., NORRBY, E. & MUFSON, M. A. (1987). Preparation and characterization of monoclonal antibodies directed against five structural components of human respiratory syncytial virus subtype B. *Journal of General Virology* **68**, 3125–3135.
- PEEPLES, M. E. & BRATT, M. A. (1982). UV irradiation analysis of complementation between and replication of RNA-negative temperature-sensitive mutants of Newcastle disease virus. *Journal of Virology* **42**, 965–973.
- PRINGLE, C. R., SHIRODARIA, P. V., GIMENEZ, H. B. & LEVINE, S. (1981). Antigen and polypeptide synthesis by temperature-sensitive mutants of respiratory syncytial virus. *Journal of General Virology* **54**, 173–183.
- SHIODA, T., IWASAKI, K. & SHIBUTA, H. (1986). Determination of the complete nucleotide sequence of the Sendai virus genome RNA and the predicted amino acid sequences of the F, HN and L proteins. *Nucleic Acids Research* **14**, 1545–1563.
- VENKATESAN, S., ELANGO, N., SATAKE, M. & CHANOCK, R. M. (1984). Structural analysis of human respiratory syncytial virus genome. In *Non-segmented Negative Strand Viruses*, pp. 27–34. Edited by D. H. L. Bishop & R. W. Compans. New York: Academic Press.
- VIDAL, S., CURRAN, J., ÖRVELL, C. & KOLAKOSKY, D. (1988). Mapping of monoclonal antibodies to the Sendai virus P protein and the location of its phosphates. *Journal of Virology* **62**, 2200–2203.
- WRIGHT, P. F., GHARPURE, M. A., HODES, D. S. & CHANOCK, R. M. (1973). Genetic studies of respiratory syncytial virus temperature-sensitive mutants. *Archives of Virology* **41**, 238–247.

---

(Received 10 June 1991; Accepted 18 November 1991)

## Effect of Changes in the Nucleotide Sequence of the P Gene of Respiratory Syncytial Virus on the Electrophoretic Mobility of the P Protein

CALLIOPE CARAVOKYRI AND CRAIG R. PRINGLE  
*Biological Sciences Department, University of Warwick, Coventry, UK*

Received March 4, 1991  
Accepted May 26, 1991

Requests for reprints should be addressed to Calliope Caravokyri, Biological Sciences Department, University of Warwick, Coventry CV4 7AL, U.K.

Key words: pneumovirus, respiratory syncytial virus, sub-type B RS virus, RSN-2 strain, P gene conservation, P protein mutants, phosphoprotein mutants, P protein structure

### **Abstract**

A consensus sequence for the P protein gene of the RSN-2 strain of respiratory syncytial (RS) virus was obtained by PCR amplification of cDNA obtained by reverse transcription. This established that the extent of sequence variation between two P genes of strains of antigenic subtype B is similar to that among A strains, confirming the conservation of P genes within subtypes and the divergence of the two antigenic subtypes of RS virus.

The P protein of RS virus exhibits anomalous electrophoretic mobility with respect to its molecular weight. In vitro transcription and translation of RSN-2 strain cDNA possessing single point mutations revealed that substitutions involving charged amino acids in the carboxy-terminal region had a marked effect on the electrophoretic mobility of the P protein.

### **Introduction**

Respiratory syncytial virus is a member of the genus *Pneumovirus* of the family Paramyxoviridae and is the most important cause of respiratory illness during human infancy. Two antigenic subtypes are recognized on the basis of reactivity

The RSN-2 strain P gene consensus sequence has been assigned the GenBank accession number M67450.

with certain anti-P protein (1) and anti-G protein (2) monoclonal antibodies. Nucleotide sequencing of the genome of a B subtype strain (18537) has confirmed the reality of the two subtypes (3-7). We report here the nucleotide sequence of the P protein gene of a second subtype B strain (RSN-2), which establishes the conservation of the P gene within subtypes and further emphasizes the divergence of the subtypes.

The P protein of RS virus is a core phosphoprotein, which, in common with the P proteins of other paramyxoviruses and rhabdoviruses, exhibits an anomalous electrophoretic mobility in relation to its molecular weight. In the case of the NS phosphoprotein of vesicular stomatitis virus (VSV), the highly negatively charged N-terminal domain is thought to hinder proportional SDS-binding, causing the aberrant (slower) migration of the protein in SDS-PAGE (8,9).

In vitro transcription and translation of variant cDNAs, generated during reverse transcription and PCR amplification, could provide an alternative approach to the analysis of epitopes and protein function in nonsegmented genome negative-strand RNA viruses, where conventional site-directed mutagenesis is not yet generally available.

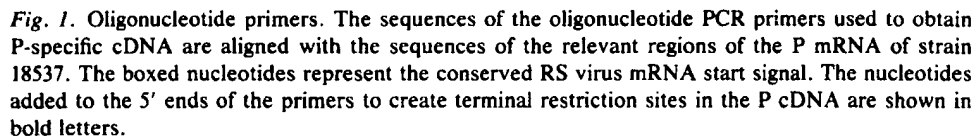
## Methods

### *Viruses and cell cultures*

The RSN-2 wild-type strain of RS virus and its temperature-sensitive mutants (10,11) were propagated in BS-C-1 cell monolayers maintained by standard methods (12).

### *Production and sequencing of cDNA clones*

cDNA clones of P mRNA from RS virus-infected cells were prepared by reverse transcription and amplification by the polymerase chain reaction (PCR). Both reactions were performed sequentially in the same reaction mixture (13) using 40 amplification cycles, each cycle consisting of 2 min at 94°C, 2 min at 55°C, and 5 min at 72°C. Total cytoplasmic RNA from cells infected with either wild type, two complementing ts mutants, or a non-ts revertant of one was used as a template for hybridization with P gene-specific primers. The primers (Fig. 1) contained terminal restriction sites to facilitate subsequent cloning, and P mRNA sequences homologous with the terminal regions of the P mRNA of the 18537 strain (7). The 5'-end specific primer comprised mostly noncoding nucleotides and was expected to be specific for the P mRNA of the RSN-2 strain, because this region and the mRNA start signal are almost exactly conserved between the two subtypes of RS virus. The 3' noncoding region is less conserved (7,14,15); therefore, the 3'-end primer was designed to be complementary to the more conserved 3'-terminal coding region.



The specificity of the PCR products was assessed by restriction enzyme mapping of recombinant plasmid DNA, following their treatment with Klenow polymerase and ligation into the unique *Sma*I site of the Bluescribe plasmid vector (Stratagene). The full-length P cDNA clones and internal fragments were subcloned into the mp18 and mp19 M13 vectors (16) for nucleotide sequencing from opposite strands by the dideoxy chain-termination method (17), using the "extended DNA sequencing" protocol suggested by the reagent supplier (BRL).

Viral cDNA clones were transcribed in vitro from adjacent bacteriophage promoters according to the method of Melton et al. (18). Quantitation of yields was carried out spectrophotometrically, and suitable amounts of the RNA preparations were used for in vitro translation. In vitro-produced uncapped RNA transcripts (150–300 ng) were translated in a nuclease-treated rabbit reticulocyte lysate (NEN). Each 25  $\mu$ l reaction contained 2  $\mu$ l of the RNA sample (in double-distilled, sterile water), 13  $\mu$ l “premix” prepared according to the manufacturer’s instructions, and 10  $\mu$ l reticulocyte lysate. Incubation was at 30°C for 2 hr, after which 25  $\mu$ l of 3x SDS-PAGE sample buffer were added prior to storage at –70°C. For immunoprecipitation, samples were diluted with 150  $\mu$ l ice-cold buffer (19) containing 2 mM PMSF and 1.2 units aprotinin (Sigma) per ml. The samples were boiled in SDS-PAGE buffer for 2–3 min and clarified before electrophoresis. SDS-PAGE, fluorography, and autoradiography were carried out by standard procedures (12).



## Results

### *The nucleotide sequence of the P gene of the RSN-2 strain of RS virus*

A consensus sequence for the P-gene cDNA-coding region of the RSN-2 strain of RS virus was compiled from sequence data of seven different PCR-derived clones. This sequence is presented in Fig. 2 as the 5' → 3'-orientated mRNA (in alignment with the equivalent region of the 18537 P mRNA). The RSN-2 sequence possesses a novel *Hind*III restriction site, which is not present in the P-gene nucleotide sequences of the A2 (3), Edinburgh (14), and Long (15) strains, which are classified in antigenic subtype A, or the 18537 (7) strain of subtype B.

The nucleotide difference creating the *Hind*III site produces one of the three amino-acid changes distinguishing the RSN-2 and 18537 strains (Fig. 2). The high level of amino-acid identity between these two subtype B strains, isolated 10 years apart, reflects the high conservation of the P protein of RS virus and is commensurate with the extent of variation among subtype A strains (Table 1). Comparison of the five P-gene coding regions shows that the P proteins are less well conserved between subtypes, and that there is relatively more intersubtype variation at the nucleotide level.

### *Sequence variation in PCR-derived clones*

Seven different P cDNA clones, one derived from P mRNA of mutant tsN1 and two (representing opposite orientations of insertion into the vector) from wild-type virus, mutant tsN19, and a non-ts revertant, were sequenced and a consensus sequence for the P cDNA coding region of the RSN-2 strain was compiled. Each of the seven independently derived P gene cDNA clones, including pairs derived from the same mRNA preparation, exhibited differences from the consensus sequence (Table 2). Despite this heterogeneity, some conclusions are possible.

A single nucleotide difference causing an amino acid change (Asn → Asp at position 217) was identified in the clone derived from mRNA from cells infected with mutant tsN1 of complementation group D. This mutant is characterized by a P protein with an aberrant electrophoretic mobility, which is unrelated to its temperature-sensitive phenotype. It is likely that this mutation is responsible for the change in electrophoretic mobility (see below).

Although several nucleotide differences occur in the two clones derived from mRNA from cells infected with mutant tsN19 (complementation group E), the nucleotide difference causing a Gly → Ser change at position 172 was common to both clones, suggesting that this difference is associated with the temperature-sensitive phenotype of this mutant. This was confirmed by immunoprecipitation and characterization of polypeptides produced by *in vitro* transcription/translation (manuscript in preparation).

		Met (1)	Glu	
1	<u>GGGGCAAUAAGUCAACAUGGAGAAGUUUGCACCUGAAUUUCAUGGAGAAGAGCAAUA</u>			RSN-2
1	<u>GGGGCAAUAAGUCAACAUGGAGAAGUUUGCACCUGAAUUUCAUGGAGAAGAGCAAACA</u>			18537
		Asp	*	
61	ACAAAGCUACCAAUUCUAGAUAUCAAUAAAGGGCAAGUUCGCAUCAUCCAAAGAUCCUA			RSN-2
61	ACAAAGCUACCAAUUCUAGAUAUCAAUAAAGGGCAAGUUCGCAUCAUCCAAAGAUCCUA			18537
		*		
121	AGAAGAAAGAUAGCAUAAUUCUGUUAACUCAUAGAUUAGAAGUAACUAAAGAGAGCC			RSN-2
121	AGAAGAAAGAUAGCAUAAUUCUGUUAACUCAUAGAUUAGAAGUAACUAAAGAGAGCC			18537
181	CGAUAAACUUCUGGCACCAACAUCAUCAAUCCAUAAGUGAAGCUGAUAGUACCCCAAG			RSN-2
181	CGAUAAACUUCUGGCACCAACAUCAUCAAUCCAUAAGUGAAGCUGAUAGUACCCCAAG			18537
		Val		
241	CCAAAGCCAACUACCCAAGAAAACCCUAGU <u>UAGCUU</u> CAAAGAAGAUUCACCCCAAGUG			RSN-2
241	CUAAGCCCAACUACCCAAGAAAACCCUAGAUAGCUUCAAAGAAGAUUCACCCCAAGUG			18537
	*	Asp		
301	ACAACCCCUUUUCUAAGUUGUACAAGAAACAAUAGAAACAUUUGAUAAACAAUGAAGAAG			RSN-2
301	ACAACCCCUUUUCUAAGUUGUACAAGAAACAAUAGAAACAUUUGAUAAACAAUGAAGAAG			18537
361	AAUCUAGCUACUCAUAUGAAGAAUAAAUGAUCAAACAAUGACAACAUACAGCAAGAC			RSN-2
361	AAUCUAGCUACUCAUAUGAAGAAUAAAUGAUCAAACAAUGACAACAUACAGCAAGAC			18537
		Met (148)		
421	UAGAUAGAAUUGAUGAAAAUUAAGUGAAUUAUAGGAUUGCUCUACAUUAGUAGUUG			RSN-2
421	UAGAUAGAAUUGAUGAAAAUUAAGUGAAUUAUAGGAUUGCUCUACAUUAGUAGUUG			18537
481	CAAGUGCAGGACCCACUUCAGCUCGCGAUGGAUUAAGAGAUGCUAUGGUUGGUCUAAGAG			RSN-2
481	CAAGUGCAGGACCCACUUCAGCUCGCGAUGGAUUAAGAGAUGCUAUGGUUGGUCUAAGAG			18537
	*			
541	AAGAAUAGAUAGAAAAUUAAGAGCGGAAGCAUUAUGACCAUAGGUUAGAGGCCUA			RSN-2
541	AAGAAUAGAUAGAAAAUUAAGAGCGGAAGCAUUAUGACCAUAGGUUAGAGGCCUA			18537
601	UGGCAAGACUUAGGAUAGGAGAAAGCGAAAAAUGGCAAAAGACACCUCAGAUGAAGUGU			RSN-2
601	UGGCAAGACUUAGGAUAGGAGAAAGCGAAAAAUGGCAAAAGACACCUCAGAUGAAGUGU			18537
		C15	Asp	W6
		▽		▽
661	CUCUAAUCCAACUCCAAAAAUGAGUGACUUGUUGGAAGACAACGAUAGUGACAAUG			RSN-2
661	CUCUAAUCCAACUCCAAAAAUGAGUAAUUUGUUGGAAGACAACGAUAGUGACAAUG			18537
		Asn		
721	AUCUAUCACUUGAUGAUUUU	740		RSN-2
721	AUCUAUCACUUGAUGAUUUU	740		18537

Fig. 2. Comparison of the nucleotide sequences of the coding region of the P mRNA of RS virus subtype B strains RSN-2 and 18537. The positions where nucleotide substitutions produce amino-acid differences are indicated. Additional silent nucleotide differences are indicated by asterisks. Also shown are the positions of the initiating methionine of the P ORF [Met(1)] and of the methionine residue used for internal initiation of translation [met(148)] during cell-free protein synthesis. The single-underlined 5' and 3' terminal sequences indicate the parts of the P sequence contained in the oligonucleotide primers. The double-underlined sequence is the unique *Hind*III restriction site. The arrowheads indicate the sites of A → G substitutions in clones C15 and W6 that produce changes in the electrophoretic mobility of the P protein.

*Table 1.* Comparison of PmRNA and protein sequences of RS virus strains of antigenic subtypes A and B

Subtype Strain (origin)	A			B	
	A2	Long	Edinburgh	18537	RSN-2
A2 (Melbourne, 1961)	—	97.2 (98.3)	97.5 (99.2)	85.5 (90.4)	85.7 (90)
Long (Baltimore, 1956)		—	97.4 (98.3)	85.0 (89.6)	84.7 (89.2)
Edinburgh (Edinburgh, 1977)			—	85.5 (90.9)	85.7 (90.5)
18537 (Washington, 1962)				—	98.8 (98.8)
RSN-2 (Newcastle, 1972)					—

The upper figures are the percent of nucleotide identities (excluding noncoding regions), and the lower figures in brackets are the percent of inferred amino-acid identities.

*Table 2.* Nucleotide differences of individual PcDNA clones and predicted changes in the respective proteins

Clone	Number of nucleotide differences	Amino-acid change
W6	1	Asp → Gly (233) <sup>a</sup>
R34	1	Ile → Val (166)
C15	1	Asn → Asp (217)
N23	3	Gly → Ser (172)
N19	4	Ser → Gly (94)
		Gly → Ser (172)
		Leu → Ser (192)

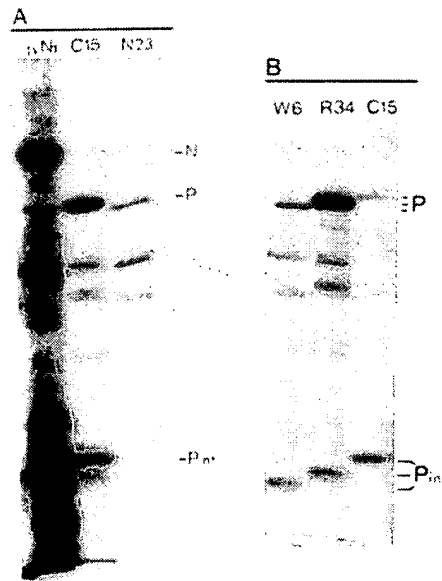
<sup>a</sup>Numbers in parentheses indicate the amino-acid position in the P polypeptide.

The other nucleotide differences were presumed to be a consequence, either of the error-prone reactions (reverse transcription and PCR amplification) that are part of the *in vitro* synthetic process, or the heterogeneity of the original mRNA preparations due to the lack of proofreading activity of the viral RNA-dependent RNA polymerase of negative-strand RNA viruses (20). This is likely to be the case for a T → C substitution at position 318 of clone N19, which is silent, although it occurs at a first codon position (TTG → CTG, both codons for Leu). These fortuitous changes, whatever their origin, provided an opportunity to assess the effect of mutations on a specific property (in this case, electrophoretic mobility) of the P protein of a negative-strand RNA virus that is not amenable to direct study by site-directed mutagenesis.

*In vitro expression of P-gene cDNA clones*

The P protein of mutant tsN1 (and its non-ts revertants) has a slower electrophoretic mobility than the wild-type protein, both in vivo (11) and in vitro (Fig. 3A). To verify that the Asn  $\rightarrow$  Asp change at position 217 was responsible for the atypical electrophoretic mobility of mutant tsN1, P gene specific RNA transcripts were produced in vitro from the tsN1-derived clone C15 and used to program cell-free protein synthesis. Figure 3A shows that the P protein obtained by in vitro transcription/translation comigrated with the P protein obtained by in vitro translation of tsN1-infected cell RNA, and both showed the characteristic mobility difference from the wild-type P protein (represented by clone N23 from tsN19 in Fig. 3A).

A substitution involving another acidic residue was observed in the sequence of the wild-type clone W6 (Fig. 2). This amino acid change was probably due to an in vitro polymerase error, since it produced an electrophoretically variant form of the wild-type P protein (Fig. 3B). The increased electrophoretic mobility of the W6 P protein correlated with loss of an acidic Asp residue and its replacement by an uncharged Gly residue, i.e., the reverse of the tsN1 situation.



**Fig. 3.** In vitro translation of the P protein from in-vitro synthesized transcripts of P cDNA clones. In panel A, translation products were immunoprecipitated with polyclonal anti-RS virus serum. Lane tsN1 contained in vitro translated polypeptides from cytoplasmic RNA isolated from tsN1-infected BS-C-1 cells. In panel B, translation products were immunoprecipitated with anti-P monoclonal antibody 8268. The P<sub>int</sub> bands are the internally initiated translation products from AUG 148 in the P ORF and exhibit the same relative mobility shifts as the corresponding P proteins.

The same relative mobility shifts are apparent in the bands labeled  $P_{int}$  in Fig. 3B.  $P_{int}$  represents the C-terminal 94 amino acids of the P protein and is produced in vitro as a result of internal initiation of translation from methionine residue 148 of the 241 amino-acid P protein (manuscript in preparation). It can be concluded from these observations that addition of an Asp in place of an uncharged residue in the carboxy-terminal region retarded the migration of the P protein, whereas replacement of Asp by an uncharged residue in the same region increased electrophoretic mobility.

### Secondary structure predictions

The secondary structures of the relevant regions of the wild type and the two mobility variants, as predicted by the algorithm of Chou and Fasman (21), are shown in Fig. 4. The region around position 233 (the site of the Asp  $\rightarrow$  Gly change) forms successive beta turns, interspersed with short regions of random coils. The substitution of Gly appears to shift the location of the second beta turn, with loss of a downstream turn, reversal of the orientation of the extreme C-terminal region, and changes in local hydrophilicity. In contrast, the Asn  $\rightarrow$  Asp change at position 217 is located within a region of random coil and has no

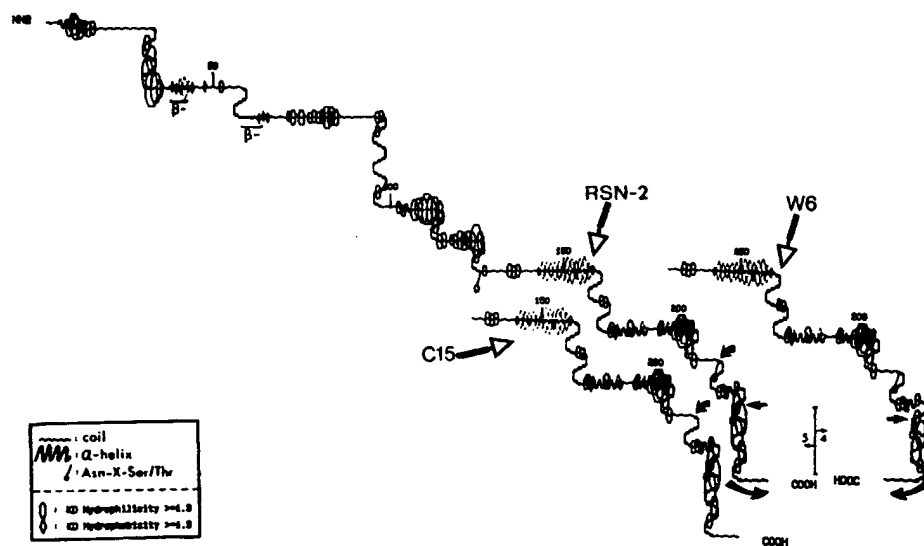


Fig. 4. Predicted secondary structure of the P protein. The secondary structure of the complete wild-type RSN-2 P protein, predicted by the method of Chou and Fassman (321), is shown together with the C-terminal regions of the C15 and W6 P proteins, where the amino-acid substitutions affecting P protein mobility are located. The two regions where a beta-sheet structure is predicted are indicated on the structure plot. Polypeptide chain reversals represent the formation of beta-turns. The number of predicted beta-turns in the extreme C-terminal region of the RSN-2 and W6 P proteins, downstream from the site of the W6 amino-acid change, is also indicated (5 and 4, respectively).

effect on the predicted secondary structure. Conversely, although two substantial changes in structure (not shown) were predicted by the Ser → Gly change at position 94 and the Leu → Ser change at position 192 (Table 2), there were no apparent changes in electrophoretic mobility of the in vitro translated P protein of cDNA clone N19. Consequently changes in the electrophoretic mobility of the P protein cannot always be associated with changes in secondary structure, at least as predicted either by the Chou and Fasman algorithm [or by Garnier plots (data not shown)].

### Discussion

The consensus sequence of the RSN-2 strain derived by amplification of cDNA obtained by reverse transcription has established that the extent of the sequence variation between two RS viruses classified in antigenic subtype B is of the same order as that among several subtype A strains, confirming the genetic divergence of the two subtypes.

The slower electrophoretic mobility of the P protein of mutant tsN1 was attributed to replacement of an uncharged Asn in the wild type by an acidic Asp in the mutant, since the P protein expressed in vitro from a tsN1-derived cDNA clone containing this change comigrated with the authentic tsN1 P protein. The replacement of an acidic Asp residue by an uncharged Gly (in the W6 P protein) had the reverse effect, i.e., increasing electrophoretic mobility. Corresponding mobility shifts were observed in the P<sub>int</sub> acidic C-terminal fragment, which, like the intact P protein, also migrates anomalously in relation to its molecular weight. This may indicate that the C-terminal region is exposed on the surface of the P protein and is at least partially responsible for the aberrant electrophoretic mobility of P.

Alterations in electrophoretic mobility due to single amino-acid differences have been described also for the phosphorylated NS protein of the New Jersey serotype of VSV (22). Two different point mutations, both involving loss of an acidic residue, were identified within the acidic N-terminal domain of the NS protein. Replacement of a wild-type Glu residue by Lys in mutant tsE1 resulted in faster migration of the NS protein, whereas a Glu → Gly substitution at an adjacent site in mutant tsE3 also produced a more rapidly migrating NS protein. In the latter case, the mobility shift was less than that associated with mutant tsE1 where the acidic residue was replaced by a basic residue. Both changes resulted in alterations to the predicted secondary structure, whereas in the case of the RS virus P mutants, it was not always possible to correlate amino-acid changes with structural effects.

In nonsegmented negative-strand RNA viruses, the analysis of the function of viral proteins by site-directed mutagenesis is as yet not a generally applicable technique. However, in certain circumstances the random sequence variation generated in the course of obtaining clones of viral genes by PCR amplification of reverse-transcribed virion or viral mRNA can be utilized in the study of viral gene products.

Some of the structural properties of the nucleocapsid-associated phosphoproteins of rhabdoviruses and paramyxoviruses can be studied in this fashion because these proteins migrate anomalously in SDS-polyacrylamide gels in relation to their actual molecular weights. Consequently mutations affecting mobility can be identified and evaluated by in vitro transcription/translation, as described above. In principle, epitope mapping could also be carried out by this approach. Indeed, the Gly → Ser substitution at position 172 in the P protein of the RSN-2 strain of RS virus has been found to abolish reactivity with a specific anti-P monoclonal antibody (manuscript in preparation).

### Acknowledgments

This work was supported by Medical Research Council grant no. PG8322715 and by a scholarship from the Greek "A.S. Onassis" Public Benefit Foundation to CC. Our thanks are due to Dr. Peter L. Collins (NIH) for provision of the sequence of the P gene of the 18537 strain prior to its publication and to Dr. Malcolm McCrae (Warwick) for assistance with the structure predictions.

### References

1. Gimenez, H.B., Cash, P., and Melvin, W.T., *J Gen Virol* 65, 963-971, 1984.
2. Anderson, L.J., Hierholzer, J., Tsou, C., Hendry, R.J., Fernie, B.F., Stone, Y., and McIntosh, K., *J Inf Dis* 151, 626-633, 1985.
3. Satake, M., Elango, N., and Venkatesan, S., *J Virol* 52, 991-994, 1984.
4. Johnson, P.R., Spriggs, M.K., Olmsted, R.A., and Collins, P.L., *Proc Natl Acad Sci USA* 84, 5625-5629, 1987.
5. Johnson, P.R. and Collins, P.L., *J Gen Virol* 69, 2623-2628, 1988.
6. Johnson, P.R. and Collins, P.L., *J Gen Virol* 70, 1539-1547, 1989.
7. Johnson, P.R. and Collins, P.L., *J Gen Virol* 71, 481-485, 1990.
8. Marnell, L.L. and Summers, D.F., *J. Biol Chem* 259, 13518-13524, 1984.
9. Chattopadhyay, D. and Banerjee, A.K., *Proc Natl Acad Sci USA* 84, 8932-8936, 1987.
10. Gimenez, H.B. and Pringle, C.R., *J Virol* 27, 459-464, 1978.
11. Pringle, C.R., Shirodaria, P.V., Gimenez, H.B., and Levine, S., *J Gen Virol* 54, 173-183, 1981.
12. Pringle, C.R., in *Virology: A Practical Approach*. Mahy, B.W.J. (ed). IRL Press, Oxford, 1985, pp. 95-118.
13. Xu, L., Harbour, D., and McCrae, M.A., *J Virol Methods* 27, 29-38, 1990.
14. Lambden, P.R., *J Gen Virol* 66, 1607-1612, 1985.
15. Lopez, J.A., Villanueva, N., Melero, J., and Portela, A., *Virus Res* 70, 249-262, 1988.
16. Yanish-Perron, C., Vieira, J., and Messing, J., *Gene* 33, 103-119, 1985.
17. Sanger, F., Nicklen, S., and Coulson, A.R., *Proc Natl Acad Sci USA* 74, 5463-5467, 1977.
18. Melton, D., Kreig, P., Rebagliati, M., Maniatis, T., Zinn, K., and Green, M., *Nucleic Acids Res* 12, 7035-7056, 1984.
19. Herman, R.C., *J Virol* 58, 797-804, 1986.
20. Holland, J.J., Spindler, K., Horodyski, F., Grabau, E., Nichols, S., and Vande Pol, S., *Science* 215, 1577-1585, 1982.
21. Chou, P.V. and Fasman, G.D., *Adv Enzymol* 47, 45-147, 1978.
22. Rae, B.P. and Elliott, R.M., *J Gen Virol* 67, 2635-2643, 1986.